Special Section on Epigenetic Regulation of Drug Metabolizing Enzymes and Transporters

Regulation of MicroRNA Expression by Rifampin in Human Hepatocytes[§]

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Received May 10, 2013; accepted August 9, 2013

ABSTRACT

Rifampin causes drug interactions by altering hepatic drug metabolism. Because microRNAs (miRNAs) have been shown to regulate genes involved in drug metabolism, we determined the effect of rifampin on the expression of hepatic miRNAs. Primary human hepatocytes from seven subjects were treated with rifampin, and the expression of miRNA and cytochrome P450 (*P450*) mRNAs was measured by TaqMan assays and RNA-seq, respectively. Rifampin induced the expression of 10 clinically important and 13 additional *P450* genes and repressed the expression of 9 other *P450* genes ($P < 0.05$). Rifampin induced the expression of 33 miRNAs and repressed the expression of 35 miRNAs ($P < 0.05$). Several of these changes were highly negatively correlated with the

rifampin-induced changes in the expression of their predicted target *P450* mRNAs, supporting the possibility of miRNA-induced regulation of *P450* mRNA expression. In addition, several other miRNA changes were positively correlated with the changes in *P450* mRNA expression, suggesting similar regulatory mechanisms. Despite the interindividual variability in the rifampin effects on miRNA expression, principal components analysis clearly separated the rifampin-treated samples from the controls. In conclusion, rifampin treatment alters miRNA expression patterns in human hepatocytes, and some of the changes were correlated with the rifampin-induced changes in expression of the *P450* mRNAs they are predicted to target.

Introduction

MicroRNA (miRNA)s are small cellular RNAs that bind to mRNAs and regulate their translation and stability. Bioinformatic predictions suggest that all of the major clinically important cytochrome P450 (*P450*) genes are targeted by miRNAs (Ramamoorthy and Skaar, 2011). In vitro functional studies have validated these predictions for some of the *P450* genes. For example, miR-27b has been shown to target *CYP1B1* (Tsuchiya et al., 2006). In vitro studies also demonstrated that

the miR-148a targets the pregnane X receptor (PXR), an important regulator of *P450* gene expression, and consequently suppresses *CYP3A4* expression (Takagi et al., 2008). In contrast, a clinical study in Han Chinese did not show an association of miR-148a expression with *CYP3A4* protein expression (Wei et al., 2013). Multiple different miRNAs target hepatic nuclear factor 4 α (HNF4A), a master regulator of many drug-disposition genes that may also be a key contributor to *P450* gene expression (Takagi et al., 2010; Ramamoorthy et al., 2012; Wang and Burke, 2013).

Altered expression of the miRNAs and genetic variants that occur in the miRNA-gene binding sites likely also contribute to the interindividual variability in drug disposition. For example, there are at least 38 genetic polymorphisms in the *P450* genes that are predicted to either create or destroy miRNA binding sites (Ramamoorthy et al., 2012). A single-nucleotide polymorphism in the miR-34a binding site of the *HNF4A* gene (rs11574744) alters the targeting of that gene by miR-34a (Ramamoorthy et al., 2012). Although it is clear that hepatic miRNA expression is altered in a variety of liver pathologies (Roderburg et al., 2011; Zhang et al., 2012; Karakatsanis et al., 2013; Liu et al., 2013), little is known about the mechanisms controlling hepatic miRNA expression or about the downstream consequences for cytochrome P450 activity.

This work was supported by the National Institutes of Health National Institute of General Medical Sciences [Grant RO1-GM088076]; and the Department of Defense predoctoral fellowship [Grant BC083078].

Parts of this work were presented as follows: Ramamoorthy A, Flockhart DA, and Skaar TC (2010) Rifampin regulates hepatic miRNA expression. *American Society for Clinical Pharmacology and Therapeutics Annual Meeting*; 2010 Mar 17–20; Atlanta, GA; and Ramamoorthy A, Liu Y, Goswami C, Philips S, Gaedigk A, Flockhart DA, Desta Z, Skaar TC (2012) Global analysis of rifampin-regulated mRNA and miRNA gene expression in primary human hepatocytes. *American Society for Clinical Pharmacology and Therapeutics Annual Meeting*; 2012 Mar 12–17; National Harbor, MD.

dx.doi.org/10.1124/dmd.113.052886.

[§]This article has supplemental material available at dmd.aspetjournals.org.

ABBREVIATIONS: HNF4A, hepatic nuclear factor 4 α ; miRNA, microRNA; P450, cytochrome P450; PXR, pregnane X receptor.

Rifampin has been an effective antimicrobial agent against Gram-negative bacteria since its introduction in the 1960s and is widely used mainly in the treatment of tuberculosis and other infections. It is known to enhance the elimination of a long list of drugs, with important clinical consequences in terms of loss or decreased drug efficacy or increased toxicity (Baciewicz and Self, 1984; Niemi et al., 2003; Chen and Raymond, 2006; Baciewicz et al., 2008). The ability of rifampin to enhance drug metabolism through induction of human P450s has been recognized since 1972 (Schoene et al., 1972). Rifampin is recognized as a pleotropic but specific inducer that alters the activity of specific P450s and other drug metabolizing and drug transporter genes, which provides the rational basis for its broad drug-drug interactions (Rae et al., 2001). The molecular mechanism by which rifampin enhances drug disposition is in part through the activation of PXR (Bertilsson et al., 1998; Lehmann et al., 1998), which induces the expression of several drug metabolizing and transport genes (Kliwer et al., 2002).

Since the evidence suggests that the *P450* genes are targets of miRNAs, we hypothesized that rifampin may also regulate hepatic miRNA expression, which in turn could regulate *P450* gene expression. In this report, we describe for the first time the effects of rifampin on the regulation of a wide range of hepatic miRNA expression.

Materials and Methods

Primary Human Hepatocytes and Drug Treatments

Human hepatocytes from seven different subjects were obtained from CellZDirect (Durham, NC). They were plated on 12-well collagen-coated plates and cultured in Williams' E medium without phenol red containing Primary Hepatocyte Maintenance Supplements (Life Technologies Corporation, Carlsbad, CA). Cultures from each subject were treated as biologic replicates ($n = 7$). All in vitro studies were performed between 72 and 120 hours from the time of hepatocyte isolation. The hepatocytes were treated for 24 hours with rifampin (10 μ M) with the corresponding vehicle control of methanol (0.01%).

RNA Isolation

Total RNA, including small RNAs, was isolated from the human hepatocytes after the rifampin treatment using the miRNeasy kit (Qiagen, Valencia, CA). The optional on-column DNase treatment was included in the purification.

MicroRNA Expression Profiling

Expression of 754 miRNAs was measured using the Taqman OpenArray Human miRNA Panel using a NT Cyclor (Applied Biosystems, Foster City, CA). RNA from each subject was analyzed on two different OpenArrays (technical duplicates). The threshold cycles were set manually based on visual inspection of the real-time amplification curves of each miRNA individually.

Bioinformatics Analysis of the miRNA Expression Data

The miRNA expression data were normalized using quantile normalization of the threshold cycle values of the miRNAs data obtained from the Taqman OpenArrays. Differential expression of the miRNAs were determined by analysis of variance, with each hepatocyte culture experiment (i.e., from a different subject) considered as a random factor; this is equivalent to a paired Student's *t* test. *P450* target genes of the differentially expressed miRNAs were predicted using Targetscan (Lewis et al., 2003).

Measurement of P450 mRNA Expression

Standard methods were used for RNA-seq library construction, EZBead preparation, and Next-Gen sequencing, based on the Life Technologies SOLiD4 system. Briefly, 2 μ g of total RNA per sample were applied for library preparation. The rRNA was first depleted using the standard protocol of

RiboMinus Eukaryote Kit for RNA-Seq (cat. no. A10837-08; Ambion, Austin, TX), and rRNA-depleted RNA was concentrated with the PureLink RNA Micro Kit (cat. no. 12183-016; Invitrogen, Carlsbad, CA) using 1 volume of lysis buffer and 2.5 volumes of 100% ethanol. After the rRNA depletion, a whole transcriptome library was prepared and barcoded per sample using the standard protocol of SOLiD Total RNA-seq Kit (cat. no. 4445374; Life Technologies). Each barcoded library was quantified by quantitative polymerase chain reaction using SOLiD Library Taqman quantitative polymerase chain reaction Module (cat. no. A12127; Life Technologies) and pooled in equal molarity. EZBead preparation, bead library amplification, and bead enrichment were then conducted using Life Technologies EZ Bead E80 System (cat. no. 4453095). Approximately 700 million library-enriched beads were deposited onto a full SOLiD4 XD slide, and finally the sequencing by ligation was performed using standard single-read, 5'-3' strand-specific sequencing procedure (50b-read) on SOLiD4.

Bioinformatic Analysis of the RNA-Seq Data

The RNA-seq data analysis includes the following steps: quality assessment, sequence alignment, and gene expression analysis.

Data Processing and Quality Assessment. We used SOLiD Instrument Control Software and SOLiD Experiment Tracking System software for the read quality recalibration. Each sequence read was scanned for low-quality regions, and if a 5-base sliding window had an average quality score less than 20, the read was truncated at that position. Any read with a length of less than 35 bases was discarded. Our experience suggests that this strategy effectively eliminates low-quality reads while retaining high-quality regions (Breese and Liu, 2013; Juan et al., 2013; Todd et al., 2013).

Sequence Alignment. We used BFAST (<http://bfast.sourceforge.net>) (Homer et al., 2009) as our primary alignment algorithm because it has high sensitivity for aligning the reads on the loci containing small insertions and deletions compared to the reference genome (hg19). We used a TopHat-like strategy (Trapnell et al., 2009) to align the sequencing reads that cross splicing junctions using NGSUtils (<http://ngsutils.org/>) (Breese and Liu, 2013). After aligning the sequence reads to a filtering index including repeats, ribosome RNA, and other sequences that are not of interest, we conducted a sequence alignment for three levels: genome, known junctions (University of California Santa Cruz Genome Browser), and novel junctions (based on the enriched regions identified in the genomic alignment). We restricted our analysis to the uniquely aligned sequences with no more than two mismatches.

RNA-Seq Differential Expression Analysis. The differentially expressed genes were identified using edgeR (Robinson et al., 2010), a Bioconductor package for differential expression analysis of digital gene expression data, based on a negative binomial distribution. To ensure the reliable gene expression measurements, we first removed the genes with less than 1 read per million mappable reads in more than half the samples in each condition. To identify the genes whose expression levels were directly affected by rifampin treatment, we further used a generalized linear model by considering the effects of individuals as a random effect (this is equivalent to a paired test). A *P* value was calculated for each gene under rifampin treatment. Benjamini and Hochberg's algorithm was used to control the false discovery rate (Benjamini and Hockberg, 1995). In this analysis, our primary focus was on the *P450* family genes.

Results

Rifampin Regulation of Hepatocyte *P450* Gene Expression. We measured the mRNA expression levels of 40 *P450* genes to determine the effects of rifampin on the hepatocyte expression of these genes. Because rifampin is known to induce the expression of a few of the *P450* genes, such as *CYP3A4*, effects on this gene allowed us to confirm the expected effects of rifampin on the hepatocytes. The expression of 10 selected *P450*s, which are clinically relevant for drug metabolism, are shown in Table 1. The expression of an additional 30 *P450* genes is shown in Supplemental Table 1. Rifampin induced the expression of several *P450*s, including *CYP3A4*, *CYP3A5*, *CYP2B6*, *CYP2C8*, *CYP2C9*, and *CYP2A6*. In contrast, *CYP2E1*, *CYP2J2*, and

TABLE 1

Gene	Fold Change ^a	<i>P</i> Value	FDR	Examples of Substrate Drugs
CYP3A4	22.1	0	0	Erythromycin, midazolam
CYP2A6	5.5	1.52×10^{-141}	2.22×10^{-138}	Letrozole, nicotine
CYP3A5	5.0	1.07×10^{-139}	1.41×10^{-136}	Tacrolimus, vincristine
CYP2C8	3.8	1.48×10^{-96}	1.39×10^{-93}	Paclitaxel, repaglinide
CYP2B6	3.6	2.01×10^{-269}	1.28×10^{-265}	Efavirenz, bupropion
CYP2C9	2.0	2.89×10^{-28}	7.03×10^{-26}	S-Warfarin, tolbutamide
CYP4F2	1.3	0.0001	0.002	S-Warfarin
CYP2D6	1.3	0.0007	0.008	Codeine, venlafaxine
CYP1A2	1.2	0.0182	0.094	Caffeine, fluvoxamine
CYP2C19	1.1	0.0315	0.136	Clopidogrel, omeprazole

FDR, false discovery rate.
^aRifampin/control.

clustering analysis of the rifampin-induced changes in miRNA expression indicated that there is substantial interindividual variability in the effect of rifampin on the miRNA expression (Fig. 2).

Correlations of miRNA and P450 mRNA Changes in Expression. To identify miRNAs that may regulate *P450* expression, we correlated the changes in miRNA expression with the changes in *P450* mRNA expression. We focused on the miRNAs that were significantly altered by rifampin (i.e., those in Table 3 and Supplemental Table 3) and correlations with *P450* genes that are predicted targets of the respective miRNAs. Because miRNAs downregulate the expression of their target genes, we first identified the top 10 negatively correlated miRNA–*P450* mRNA pairs (Table 4). For several of these pairs, the correlation coefficients were highly negative, providing additional support for direct regulation by the miRNAs. Interestingly, these were all in genes that were not regulated by rifampin ($P > 0.05$). Because none of the clinically relevant *P450* genes appeared on this list, we evaluated the 10 clinically relevant *P450* genes for either positive or negative correlations (Table 5; correlations with all of the *P450*s are in Supplemental Table 4). Nearly all of the correlations with the clinically relevant *P450* genes were positive correlations. Interestingly, the correlations were mostly positive for the *P450* genes that were induced by rifampin whereas the negative correlations were mostly with *P450* genes that were minimally or not significantly altered by rifampin. In addition, although the expression of the *P450* genes in Table 4 were not statistically significantly altered by rifampin, they were all correlated with each other (Supplemental Table 2).

Discussion

Our studies provide evidence that drugs can substantially alter the expression patterns of miRNAs in hepatocytes, as demonstrated by

TABLE 2

[illegible]

Fig. 1. Effect of rifampin on global miRNA expression in primary human hepatocytes. Each dot is a different miRNA and represents the mean effect size and P value from $n = 7$ different subjects. The x -axis is the effect size on a $\log(2)$ scale as the ratio of the expression in the rifampin/control treated hepatocytes. The y -axis is the $-\log(10)$ of the P value for the comparison of the expression in the rifampin and control treated hepatocytes. Red dots are those miRNAs with $P < 0.01$; orange dots are $P > 0.01$ and $P < 0.05$; green are $P > 0.05$. The miRNAs labeled are some of those with $P < 0.05$ and effect size >2 -fold up- or down-regulation. The specific effect sizes and P values for the miRNAs are listed in Table 3 and Supplemental Table 3. hsa, Homo sapiens.

rifampin for which we observed both positive and negative effects on miRNA expression. The alteration of only some of the expressed miRNAs provides a mechanism for regulation of specific *P450* genes. Because these miRNAs are predicted to target many of the *P450* genes (Ramamoorthy and Skaar, 2011), it is possible that part of the rifampin effect on the expression of the drug metabolizing enzyme genes may be through altered miRNA expression patterns. Because miRNAs can also block mRNA translation in the absence of effects on mRNA expression, it is likely that there are additional levels of regulation that were not detected in our investigation. The elucidation of the specific

effects of individual miRNAs will come from future experiments focused on the transfection of miRNAs mimics and antagonists to decipher the contribution of individual miRNAs to the overall effect of the rifampin. However, it is possible that the rifampin regulation of miRNAs, such as miR-34a and miR-148a, affects drug metabolism, as other studies have shown that these miRNAs affect *P450* genes directly and indirectly through transcription factors such as PXR and HNF4A (Takagi et al., 2008, 2010; Ramamoorthy et al., 2012; Wang and Burke, 2013). The highly negative correlations of some of the miRNAs with their predicted targets (Table 4) provide strong support for this mechanism for some of the *P450* genes.

It was interesting to note that for the *P450* genes that were highly induced by rifampin (e.g., *CYP3A4*, *CYP2B6*), the correlations with miRNAs appeared to be mostly positive correlations. There could be several explanations for this. First, both the *P450* and the miRNA genes may have similar regulatory mechanisms in their promoters, such as PXR response elements. Second, the lack of detectable negative correlations may be because the induction of the *P450* gene was so strong (e.g., >20 -fold for *CYP3A4*) that the negative effects of the induced miRNAs are masked by the strong transcriptional induction. Third, it is conceivable that the miRNAs are important for regulating basal expression of these *P450* genes, but they are relatively unimportant to the drug-induced levels. Examination of these two possibilities would require the analysis of large numbers of liver or hepatocyte samples to identify significant correlations with basal expression levels or the exogenous expression of the miRNAs in hepatocytes. These are the focus of ongoing studies with individual and combinations of miRNAs. Fourth, it is possible that the miRNAs have been induced later than the *P450* genes (i.e., closer to the 24-hour time point) and there has not yet been time for the miRNA to cause degradation of the mRNA.

Several other miRNAs have been shown to target genes involved in the regulation of *P450* gene expression. We have demonstrated that miR-449 can regulate the *HNF4A* 3'-untranslated region (Ramamoorthy

TABLE 3

miRNAs regulated by rifampin in hepatocytes

Includes only miRNAs with $P < 0.01$.

miRNA	P Value	Fold Change ^a
Upregulated		
miR-886-3p	0.0006	2.3
miR-26b	0.0007	1.3
miR-21	0.0020	1.4
miR-218	0.0028	1.8
miR-29c	0.0029	1.2
miR-25	0.0045	1.3
miR-194	0.0056	1.2
Downregulated		
miR-27a	0.0015	-3.0
miR-135a	0.0025	-18.9
miR-149	0.0028	-1.6
miR-671-3p	0.0034	-4.0
miR-95	0.0040	-1.8
miR-1303	0.0042	-3.5
miR-200b#	0.0049	-2.3
miR-331-5p	0.0051	-7.2
miR-1180	0.0082	-12.6

^aRifampin/control. The ratios of the downregulated genes have been converted to negative values by taking the negative reciprocal of the rifampin/control.

Fig. 2. Hierarchical clustering heat map of the rifampin-induced changes in hepatocyte miRNA expression. Hierarchical clustering was performed on the expression patterns of the miRNAs that were significantly altered by rifampin ($P < 0.05$) and had an effect size of at least a 50% change. Green is low expression, and red is high expression. Each of the seven rows are the data from the seven different hepatocyte preparations.

et al., 2012), and miR-27b is known to regulate *CYP1B1* (Tsuchiya et al., 2006). MicroR-24 indirectly regulates *CYP1A1* through its effects on the Aryl hydrocarbon receptor nuclear translocator (*ARNT*) (Oda et al., 2012) and *HNF4A* (Takagi et al., 2010). However, none of these were regulated by rifampin in our studies.

We also provide herein a comprehensive RNA-seq analysis of the rifampin-induced changes in *P450* gene expression. As expected, several of the *P450* genes were induced to a large extent (e.g., *CYP3A4*, *CYP2B6*, *CYP2A6*). Interestingly, several of the genes were downregulated by rifampin, such as *CYP2E1* and *CYP4A11*. Although rifampin is widely viewed as an inducer of gene expression, these data confirm the prior studies (Rae et al., 2001) that indicated that it is also able to notable decrease the expression of a number of important genes. There were also several genes that were minimally altered by rifampin (e.g., *CYP2D6*, *CYP2C19*, *CYP1A2*). The induced gene expression is mediated at least in part by the rifampin activation of PXR; however, the mechanism of the downregulation of the *P450* genes is unknown.

Correlations between rifampin-induced changes in expression of the different *P450* mRNAs were also notable. Several of the induced

genes were highly positively correlated (e.g., *CYP2A6*, *CYP2B6*, *CYP3A4*). However, the extent of induction of *CYP3A5*, which was induced 5-fold by rifampin, was negatively correlated with all of the other clinically important genes. This indicates that the hepatocytes with the greatest induction of *CYP3A5* had the least induction of the other clinically important *P450* genes. Furthermore, some of the *P450* genes that were minimally induced had highly correlated changes in expression (e.g., *CYP2C9* and *CYP2D6*). Because the expression went up in some subjects and down in others, the mean is a small effect size with not very significant *P* values. However, they are correlated because the variability in these genes follows a similar pattern in the different subjects. For example, in subjects in whom *CYP2C9* expression

TABLE 5
miRNAs correlated with clinically important P450 genes

miRNA	P450 Gene	R ^a
hsa-miR-590-5p	CYP2B6	0.69*
hsa-miR-27a	CYP4F2	0.63**
hsa-miR-148a	CYP2B6	0.56
hsa-miR-29b	CYP2B6	0.54
hsa-miR-27a	CYP3A4	0.53
hsa-miR-135a	CYP4F2	0.50
hsa-miR-203	CYP1A2	-0.49
hsa-miR-200a	CYP4F2	0.48
hsa-miR-106b	CYP3A4	0.41
hsa-miR-182	CYP1A2	0.37
hsa-miR-20a	CYP3A5	0.36
hsa-miR-200a	CYP2B6	0.35
hsa-miR-21	CYP2B6	0.35
hsa-miR-200a	CYP2C8	0.30
hsa-miR-20a	CYP1A2	0.29
hsa-miR-25	CYP2C8	-0.26
hsa-miR-106b	CYP4F2	0.25
hsa-miR-590-5p	CYP3A5	-0.25

^aGene pairs with correlations of at least ± 0.25 were included. Data are ordered from top to bottom by the absolute value of the R value.

* $P < 0.10$; ** $P < 0.15$.

TABLE 4

P450-miRNA pairs with highly negative correlations consistent with downregulation of the P450 gene by the miRNAs

miRNA	P450 Gene	R	P Value
hsa-miR-200b	CYP1B1	-0.94	<0.01
hsa-miR-15b	CYP2U1	-0.86	0.01
hsa-miR-15b	CYP1B1	-0.84	0.02
hsa-miR-429	CYP1B1	-0.81	0.03
hsa-miR-221	CYP1B1	-0.77	0.04
hsa-miR-200b	CYP4V2	-0.77	0.04
hsa-miR-195	CYP4V2	-0.72	0.07
hsa-miR-34a	CYP11A1	-0.66	0.11
hsa-miR-15b	CYP4V2	-0.66	0.11
hsa-miR-20a	CYP4V2	-0.64	0.12

was increased, *CYP2D6* expression also increased. This suggests that the mechanisms that contribute to the interindividual variability in these genes are similar.

We do acknowledge some limitations to this study. First, one of the factors that may contribute to the interindividual variability in rifampin response is genetic differences between hepatocyte donors. For example, single-nucleotide polymorphisms in PXR, which binds rifampin and mediates at least some of the effects on P450 gene expression, could contribute to this variability. Because of the small sample size in this study, PXR was not genotyped, but this should be considered for future studies. Second, we only analyzed one time point. Useful information may be discovered if additional time points are analyzed. Lastly, miRNAs can alter mRNA translation without altering the transcript levels, so important effects of the miRNAs on the P450 protein amounts that are not detected by our mRNA analyses may have been missed.

In conclusion, our studies clearly show that the miRNA expression profiles in human hepatocytes are regulated by drug therapy. Using rifampin, a drug well known to alter hepatic drug metabolism, we have presented a comprehensive analysis of its effects on miRNA expression. Furthermore, through a comprehensive analysis of the expression of the 40 *P450* genes, our studies show that rifampin has a broad spectrum of effects on these different *P450* genes and identified specific correlations among these genes that will be useful in guiding future experiments to further elucidate the regulatory mechanisms that control their increased and decreased expression.

Authorship Contributions

Participated in research design: Ramamoorthy, Liu, Desta, Gaedigk, Li, Flockhart, Skaar.

Conducted experiments: Ramamoorthy, Philips.

Performed data analysis: Ramamoorthy, Liu, Philips, Li, Goswami, Lin, Skaar.

Wrote or contributed to the writing of the manuscript: Ramamoorthy, Liu, Desta, Gaedigk, Li, Flockhart, Skaar.

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